Cell Metabolism
Review

Unimolecular Polypharmacy for Treatment of Diabetes and Obesity

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Many complex diseases have historically proven to be defiant to the best mono-therapeutic approaches. Several examples of combination therapies have largely overcome such challenges, notably for the treatment of severe hypertension and tuberculosis. Obesity and its consequences, such as type 2 diabetes, have proven to be equally resistant to therapeutic approaches based on single medicines. Proper management of type 2 diabetes often requires adjunctive medications, and the recent registration of a few compound mixtures has set the precedent for combinatorial treatment of obesity. On the other hand, double or triple therapeutic combinations are more difficult to advance to regulatory approval than single molecules. More recently, several classes of novel unimolecular combination therapeutics have emerged with superior efficacy than currently prescribed options and pose the potential to reverse obesity and type 2 diabetes. Here, we summarize the discovery, pre-clinical validation, and first clinical test of such peptide hormone poly-agonist drug candidates.

A Call for Action

According to the International Diabetes Federation (IDF), global diabetes prevalence is nearly 10% among adults, with deaths attributable to diabetes anticipated to rise by >50% over the next decade. Type 2 diabetes (T2D) accounts for more than 90% of the disease (Guariguata et al., 2014). Although not everybody with excess body weight develops T2D, and not everyone suffering from T2D is overweight, the increased T2D prevalence is predominantly enhanced by excess body weight, with two billion adults and forty million children currently overweight or obese, according to estimates from the World Health Organization (WHO). As disease incidence steadily grew throughout the last three decades, research efforts to effectively address it intensified. This period is characterized by unprecedented advances in the understanding of the cellular processes, molecular targets, and neuroendocrine signals controlling metabolism. Nonetheless, initial drug candidates for obesity have limited efficacy and/or cause unacceptable adverse effects (Adan, 2013; Rodgers et al., 2012; Scheen and Van Gaal, 2014). We propose that synergistic integration of multiple hormones of differentiated mechanisms to single molecules may conquer the endogenous systems that defend increased body weight and have limited the effectiveness in agents employing a single mechanism of action.

A Complex Puzzle

Energy balance is central to survival and as such it is intricately regulated. In a relative sense, the long-standing strategy to drive weight loss has been conceptually simple and constituted by increased caloric use (energy expenditure) or reduced caloric consumption (energy restriction). More than a century ago, thyroid extracts were recognized for their weight-lowering properties by increasing caloric burn. Indeed, the first generation of weight-loss medications of the 1950s and 1960s, which included sympathomimetics, amphetamines, and chemical uncouplers, lowered body weight by increasing metabolic rate. However, sustained use of such agents resulted in severe adverse cardiovascular effects that terminated their use. In contrast, agents targeting reduction in food intake, including serotonergics, dopaminergics, and endocannabinoid antagonists have largely been safer, but of relatively lesser efficacy when compared to the aforementioned thermogenic drugs. However, these anorectic agents are not without their own limitations, including prominent cardiovascular effects and psychiatric symptoms resulting from action within the CNS.

More recently, research in the fields of metabolism and neuroscience has revealed a complex entwinement of satiety and thermogenic signaling cues that are reciprocally coordinated between peripheral tissues and metabolic control centers in the CNS. These molecular findings have paved the way for drug discovery in the modern era. The identification of the adipose tissue-derived hormone leptin 20 years ago, and its unprecedented effectiveness to induce satiety and increase energy expenditure (Farooqi et al., 1999; Halaas et al., 1995; Heymsfield et al., 1999; Zhang et al., 1994), generated hope that this single adipokine could address obesity in a similar fashion to the way insulin transformed the treatment of juvenile-onset diabetes. Unfortunately, the weight-lowering efficacy of pharmacological leptin in conventional obesity is dramatically blunted. Nonetheless, the discovery of leptin profoundly transformed modern obesity research and promoted interest for circulating signals that, similar to leptin, simultaneously target brain circuits governing feeding and cellular processes that control energy metabolism in peripheral tissues (Bates et al., 2003; Elias et al.,

1999; Schwartz et al., 1997; Tschöp et al., 2000). Genome-wide searches for associations between obesity and single-gene mutations confirmed a central role for the brain, as most known "obesity genes" appear to be expressed or function predominantly in the CNS (Locke et al., 2015). However, translation of these novel findings into effective therapies for obesity and T2D has proven difficult. Adverse effects often accompany metabolic benefits (Christensen et al., 2007; Ettinger et al., 2003; Greenfield et al., 2009), or underwhelming weight lowering is observed (Allison et al., 2012; Davidson et al., 1999; Plodkowski et al., 2009; Smith et al., 2010; Vilsbøll et al., 2012). The few drugs that have been approved for the treatment of T2D offer a meaningful but limited impact on adiposity. These include inhibitors of sodium-glucose co-transporter 2 (SGLT2) (Barnett, 2013) and analogs of the incretin hormone glucagon-like peptide 1 (GLP-1) (Vilsbøll et al., 2012). Additionally, a few small-molecule drugs have recently gained approval for weight management, including selective serotonin receptor agonists (lorcaserin) and unique combinations of a sympathomimetic with an anticonvulsant (phentermine/topiramate), as well as an opioid antagonist with an antidepressant (naltrexone/bupropion). However, mean body weight reduction remains well below 10% compared to placebo-treated controls with all of these therapies, although a high dose of the GLP-1 analog liraglutide is starting to push prior limits in responsive patients (Pi-Sunyer et al., 2015).

Lessons from Bariatric Surgery

Although the goal of translating novel molecular discoveries in metabolic control to useful medicines is far from being attained, there is empirical evidence that effective targets do exist. The effectiveness of specific surgical procedures inducing weight loss and improved glycemic control in morbidly obese patients is inspirational. Procedures such as Roux-en-Y gastric bypass or vertical sleeve gastrectomy not only sizably reduce fat mass, but also resolve insulin resistance and T2D in most patients. Interestingly, improvements in glucose metabolism precede significant weight loss (Stefater et al., 2012). These observations, along with mechanistic insights gained from bariatric surgery in animal models of obesity and insulin resistance, suggest that re-programming of neuroendocrine signals rather than changes in mechanical function of the gut (i.e., adjusted stomach size and/or impaired calorie absorption per se) are predominantly responsible for the substantial improvements in caloric, glucose, and lipid metabolism (Adams et al., 2012; Buchwald et al., 2009; Seeley et al., 2015). Most bariatric surgeries significantly alter circulating levels of hormones such as GLP-1 (Korner et al., 2007), glucose-dependent insulin-tropic peptide (GIP) (Näslund et al., 1998), peptide YY (PYY) (Chan et al., 2006), bile acids (Patti et al., 2009; Ryan et al., 2014), glucagon (Jørgensen et al., 2013), ghrelin (Cummings et al., 2002), and fibroblast growth factors (FGFs) (Jansen et al., 2011), among many others. Proof for these adjusted endocrine responses, either in support or opposition to their singular, combined, causal, or consequential involvement in metabolic benefits of weight loss surgeries, remains the subject of ongoing investigations.

An Emerging Path

Despite the metabolic complexity in body weight management, modern obesity research has made steady progress. Both behavioral and cellular processes relevant for body weight homeostasis and systemic metabolism appear to be continuously co-regulated by finite brain control centers, including specific neural circuits in the hypothalamus. Moreover, it now appears that the brain is just as involved in the pathogenesis of obesity and T2D as traditional target organs such as the pancreas, skeletal muscle, liver, and adipose tissue. History has taught us that direct pharmacological intervention of CNS neurotransmission is difficult to limit to just those brain circuits controlling body weight and metabolism, and therefore they are susceptible to severe adverse effects (Dietrich and Horvath, 2012). Nevertheless, it seems plausible that a sustained readjustment of CNS control centers to normalize body weight and glucose metabolism of obese and diabetic patients might be safely achieved. Gastric bypass surgery may be precedent setting by instating a different neuroendocrine multisignal code. Using "mother nature's tool kit" of afferent hormones, pharmacological mimicry of this integrated and adjusted enteroendocrine response may offer an effective and relatively safe approach to modulate brain circuits controlling body weight and systemic metabolism through relays to relevant peripheral tissues. The overarching questions are how many of these hormonal activities must be manipulated simultaneously, and whether these patterns vary across multiple forms of human obesity. Ideally, the integration of multiple actions into single drugs lessens the regulatory challenge and minimizes the number of mixtures that might ultimately be required, while producing coordinated actions in target tissues that provoke synergistic effects. Nonetheless, physical combinations of two independent mono-agonists do present some advantages, most notably the flexibility in adjusting activity ratios.

Against All Odds: Glucagon for Obesity

The seminal obstacle in the development of effective anti-obesity drugs is the fact that CNS control centers respond to decreased food intake by decreasing energy expenditure or, conversely, increasing appetite when confronted with elevated metabolism, as is the case with exercise. Therefore, combining a satiety-inducing hormone with a factor that promotes calorie burning into a single unimolecular entity should create a drug candidate of increased efficacy. This strategy seems more straightforward to assess in macromolecules, where size is not a limitation to achieving high potency and balanced pharmacology. Additionally, the action of large molecules is generally more restrictive than conventional small molecules, and subsequent drug metabolism is rarely a dose-limiting element to pharmacology.

The starting point in our efforts to generate safe and potent poly-agonists for the treatment of obesity and T2D was GLP-1. The choice of GLP-1 was justified by a number of reasons. GLP-1 serves a role in the mediation of bariatric surgery benefits (Habegger et al., 2013a, 2014; Salehi and D'Alessio, 2014; Salehi et al., 2011). GLP-1 agonists target both the CNS and pancreas to promote satiety and insulin secretion, which has led to regulatory approval for several GLP-1-based therapies in the treatment of T2D. Currently prescribed GLP-1 mono-agonists provide a meaningful yet insufficient body weight loss in most obese patients, primarily through anorectic or satiation properties (Vilsbøll et al., 2012). Increasing the dose of most GLP-1 agonists enhances weight loss, as with high-dose liraglutide (Pi-Sunyer

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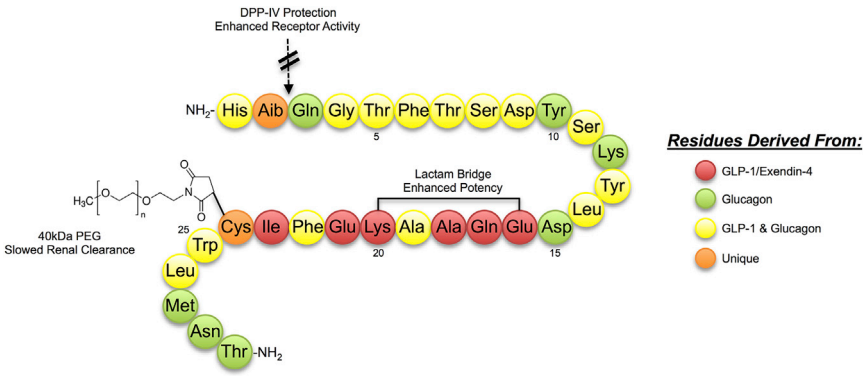

Figure 1. Structure of GcgR/GLP-1R Co-agonist

Amino acid sequence of the GcgR/GLP-1R co-agonist (Day et al., 2009). Residues derived from GLP-1 or exendin-4 are depicted in red, residues derived from glucagon are depicted in green, residues shared between GLP-1 and glucagon are depicted in yellow, and unique resides are depicted in orange. Aib, aminoisobutyric acid. Additional chemical engineering, including an *i, i+4* side chain macrocyclization from residues Glu^{16} to Lys^{20} and a 40 kDa polyethylene glycol (PEG) moiety at Cys^{24}, is also represented with chemical structures.

et al., 2015), but magnifies a dose-dependent increase in adverse gastrointestinal effects and acute tachycardia (Marino et al., 2014). Combining GLP-1 agonism with an independent thermogenic factor seems intuitively attractive to propel greater body weight loss. In addition, less reliance on GLP-1-mediated signaling to drive greater body weight loss can circumvent the adverse events that preclude the use of such mono-agonists at higher doses.

In our search for a complementary component that promotes weight loss through biochemical and physiological mechanisms that are distinct from GLP-1, we were intrigued by reports from as early as the 1950s that detailed the chronic actions of the pancreatic hormone glucagon to increase lipolysis and thermogenesis (Davidson et al., 1957; Joel, 1966; Kuroshima and Yahata, 1979). However, integrating glucagon action into agents directed to patients with impaired glycemic control was a radical idea, as common belief held that glucagon was part of the problem promoting T2D (Müller et al., 1970). The logic was one of "fighting fire with fire" at a molecular level, and to do so, GLP-1 agonism was pursued as a means to buffer against the inherent diabetogenic risk of unopposed glucagon pharmacology, but also to provide supplemental efficacy by an independent weight-lowering mechanism.

A series of single-molecule glucagon receptor (GcgR)/GLP-1 receptor (GLP-1R) dual agonists were generated using glucagon as a template sequence to which chemical modifications were introduced. Extensive structure-activity relationship (SAR) profiling, as well as knowledge gained from chimeric peptides used to map receptor recognition epitopes (Hjorth et al., 1994), resulted in a hybrid peptide of mixed glucagon and GLP-1 sequence that was structurally supplemented to prevent proteolysis and delay metabolic clearance (Figure 1) (Day et al., 2009). The resulting peptides were of comparable structure to glucagon and GLP-1, but of balanced agonism at each receptor and comparable inherent potency to native hormones. This mixed agonist sizably lowered body weight of diet-induced obese mice (Clemmensen et al., 2014; Day et al., 2009, 2012), and the magnitude of weight loss was dependent on the percentage of glucagon activity. The body weight loss is almost entirely due to decreased body fat mass, and there was no appearance of hyperglycemia until the GLP-1 activity was reduced to a level that was appreciably less than glucagon. In opposition to the prevailing logic, these GcgR/GLP-1R dual agonists safely improved glucose tolerance, hyperinsulinemia, hepatic steatosis, and body weight to a greater extent than possible with either mono-agonist in preclinical models of obesity and those with hyperglycemia. Genetic loss-of-function studies using GLP-1R knockout ($GLP\text{-}1R^{-/-}$) mice proved that the superior performance of these dual GcgR/GLP-1R co-agonists was indeed dependent on mixed pharmacology and not simply a result of enhanced GLP-1 potency. Based on in vitro data, it had also been suggested that oxyntomodulin, another gut peptide involved in metabolic control (Dakin et al., 2001), would potentially represent an endogenous GLP-1/glucagon co-agonist. However, oxyntomodulin is a weak, imbalanced agonist at both receptors. Further, oxyntomodulin has been shown to have minimal effects on energy homeostasis in $GLP1\text{-}R^{-/-}$ mice, while effects are preserved in GcgR knockout ($GcgR^{-/-}$) mice (Baggio et al., 2004), indicating that oxyntomodulin is not an endogenous unimolecular dual agonist.

The metabolic improvement resulting from the massive decrease in excess body fat, together with the known beneficial metabolic actions of GLP-1, overrides the inherent diabetogenic property of glucagon agonism. These findings have been confirmed by independent investigators using an oxyntomodulin-based analog with improved pharmacokinetic parameters, which also showed enhanced metabolic endpoints compared to a suitably matched GLP-1 agonist (Pocai et al., 2009). Furthermore, the superior actions of GcgR/GLP-1R co-agonists translate from rodents to nonhuman primates (Figure 2) (Lao et al., 2013), but clinical data have yet to publish. Although the optimal activity ratio to achieve maximum metabolic benefits with minimized hyperglycemia in rodents appears to be approximately 1:1 (Day et al., 2012), the preferred activity ratio for human benefit is unknown and likely to vary given the broad presentation of disease. The exact molecular action that governs the action profile of the dual agonist is still being investigated, as well as the relative virtue in accomplishing the mixed pharmacology in a single peptide. One important component has already been discovered: glucagon is a secretagogue for fibroblast growth factor 21 (FGF21), an endogenous protein acting at the level of the brain, liver, and adipose tissue to decrease body weight and improve dyslipidemia (Habegger et al., 2013b), which itself has demonstrated translational benefits as an anti-obesity therapy (Adams et al., 2013; Gaich et al., 2013; Kharitonenkov et al., 2007; Talukdar et al., 2016). Clinical trials with these GLP-1/glucagon mixed agonists in multiple forms are ongoing (Table 1), but results are only beginning to be

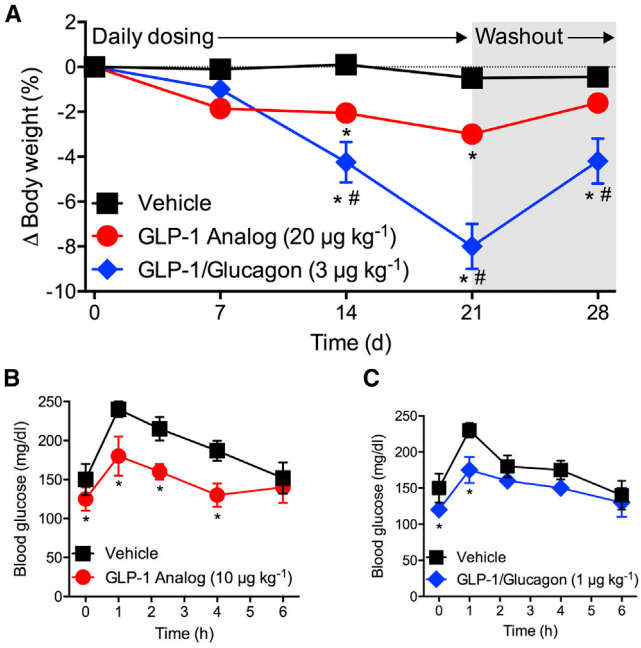

Figure 2. Translational Superiority of GcgR/GLP-1R Co-agonism versus GLP-1R Mono-agonism

(A) Effects of a 7-fold lower dose of a GcgR/GLP-1R co-agonist (blue diamonds) versus liraglutide (red circles) to lower body weight in diet-induced obese rhesus monkeys following daily subcutaneous injections at the indicated doses.

(B and C) Effects on a meal tolerance test following 7 days of subcutaneous injections of (B) liraglutide (red circles) or (C) a 10-fold lower dose of a GcgR/GLP-1R co-agonist (blue diamonds) in diabetic rhesus monkeys. *p < 0.05 comparing compound injections to vehicle. #p < 0.05 comparing co-agonist injections to mono-agonist injections.

Error bars are means ± SEM. Adapted from Lao et al. (2013).

revealed publically. A preliminary study in humans indicates that co-infusion of native glucagon and GLP-1 amplifies anorectic and energy expenditure effects without any detectable adverse effects (Cegla et al., 2014; Tan et al., 2013). Amazingly, GcgR agonism has now emerged as a provocative tool that is now established to maximize weight loss via increased thermogenesis in concert with a complementary pharmacology that enhances efficacy and lessens toxicity (Campbell and Drucker, 2015; Habegger et al., 2010; Heppner et al., 2010). Simultaneously, GcgR antagonism is still pursued by others for the treatment of hyperglycemia, but concerns pertaining to stabilization of hepatic fat, pancreatic α cell hyperplasia, and elevated lipids have led to the prospect of mixed GLP-1R agonism with glucagon antagonism (Claus et al., 2007; Pan et al., 2006).

Get a GIP: "Twincretins"

Inspired by the successful development and validation of GcgR/GLP-1R co-agonists as anti-obesity therapeutics, we added strategies to generate another co-agonist initially geared toward improved treatment of hyperglycemia in T2D. Aiming to enhance the glycemic benefits of GLP-1, the structurally related second member of the two principle incretin hormones, GIP, was selected as the second component in a new series of dual agonists (Finan et al., 2013). These co-agonist peptides are similar in size and structure to the two native incretins. These peptide hybrids bind and activate the GLP-1R and GIP receptor (GIPR) with balanced near-equal activity and include chemical moieties that protract in vivo time action (Figure 3A) (Finan et al., 2013). These dual GIP/GLP co-agonists exhibit superior in vivo efficacy in mice, rats, nonhuman primates, and humans when compared to equimolar mono-agonists (Finan et al., 2013). In particular, insulin secretion and glucose tolerance improved and, despite double incretin action, neither chronic hyperinsulinemia nor hypoglycemia was observed (Finan et al., 2013). The molecular actions that govern the synergistic weight loss are being explored, but enhanced anorectic actions and hormonal sensitivity appear to mediate the body weight effects. Measures of gastrointestinal motility in preclinical studies and in the first human trials suggested that adverse events were reduced for the co-agonist as compared to GLP-1 mono-agonist treatment (Finan et al., 2013).

While seemingly intuitively obvious given the endogenous and complementary roles that GIP and GLP-1 serve as incretins, the path to GIP agonism has been tortuous. A central limitation is the disproportionate reduction in GIP efficacy relative to GLP-1 in persistent hyperglycemia (Nauck et al., 1993). While these results point to GLP-1 as the preferred singular choice, our logic has never been limited to just one, and the attraction of using GLP-1 within a co-agonist to restore proper glycemic control, and concomitantly GIP activity (Højberg et al., 2009), was fundamental to our interest in the discovery and advancement of dual incretin co-agonists. The discovery of the GIPR/GLP-1R co-agonists and investigation in animals and humans have shed new light on GIPR agonism. Prior to the observations made with these novel dual agonists, common opinion based on global GIPR knock out (*GIPR*$^{-/-}$) mouse models (Miyawaki et al., 2002) and chemical GIPR antagonism studies (McClean et al., 2007) using what is now an inappropriately characterized GIPR antagonist (Sparre-Ulrich et al., 2016) had predicted that GIPR agonism promoted body weight gain. Additionally, single-dose clinical studies have reported no additional virtue to GIPR agonism in T2D (Mentis et al., 2011). Nonetheless, GIP-R/GLP-1R dual agonists actually drive more body weight loss than mono-agonists. The subsequent behavior of other GIPR/GLP-1R co-agonists has validated our initial report and is currently being pursued in multiple clinical trials within the pharmaceutical industry (Table 1) (Finan et al., 2015a). The recent increasing attention for GIP has also emphasized how little is known about the molecular signaling and action profile of this hormone (Finan et al., 2016), including the potential of undesired side effects arising from GIPR agonism (Berglund et al., 2016; Gögebakan et al., 2015). One of the benefits of co-agonism is represented by the fact that synergistic action as observed in these "twincretins" offers the opportunity for the use of very low dosing, thereby lowering the risk of side effects and potentially increasing the therapeutic window.

It should be noted that although it cannot be definitively overruled, the design of these sequence-mixed dual agonists is not based on the concept of simultaneous binding of two different cognate receptors at the same target cell, as would be the case of a co-agonist multimer or fusion peptide. Instead, the goal for both GcgR/GLP-1R and GIPR/GLP-1R co-agonists is to bind to either one or the other receptor with equally balanced preference, leading to equal relative levels of occupancy at each receptor. Importantly, this comparative receptor activity can be

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Table 1. Competitive Landscape of Proglucagon-Based Mixed Agonists

Name	Company	Actions	Protraction	Status	Latest Clinical Results
LY2944876 / TT-401	Eli Lilly / Transition Therapeutics	GLP-1, Gcg	weekly	phase 2[a]	doses: 10, 15, 30, 50 mg
					frequency: weekly
					duration: 24 weeks
					A_{1C} (50 mg): −1.4%
					BW (50 mg): −3.3 kg
					Keystone Meeting, April 2016
HM12525A	Janssen / Hanmi Pharmaceuticals	GLP-1, Gcg	weekly	phase 1	doses: 0.25–4.0 nmol/kg
					frequency: weekly
					duration: 56 days
					ADA Scientific Sessions 2015
SAR425899	Sanofi	GLP-1, Gcg	daily	phase 1	doses: NA
					frequency: daily
					duration: 28 days
					A_{1C} (highest): −0.6%
					BW (highest): −5.5 kg
					Keystone Meeting, April 2016
MEDI0382	AstraZeneca / MedImmune	GLP-1, Gcg	–	phase 1	undisclosed
PSA-Oxyntomodulin	Xenetic Biosciences	GLP-1, Gcg	PSA	phase 1[b]	doses: 0.25–1.5 mg/kg
					duration: 28 days
MOD-6031	OPKP Biologics / Prolor Biotech	GLP-1, Gcg	PEG	–	doses: 20, 50, 100, 150, 200 mg
					frequency: ~monthly
					duration: 30 days
ZP2929	Zealand	GLP-1, Gcg	daily	phase 1	undisclosed
VPD-107	Spitfire Pharma	GLP-1, Gcg	EuPort	preclinical	NA
Undisclosed	Merck	GLP-1, Gcg	–	unknown	NA
Undisclosed	Zealand / Boehringer Ingelheim	GLP-1, Gcg	weekly	unknown	NA
Liraglutide + NN9030	Novo Nordisk	GLP-1 + Gcg	acyl	preclinical	NA
NN9709 / MAR709	Novo Nordisk / Marcadia	GLP-1, GIP	acyl	phase 2	undisclosed
SAR438335	Sanofi	GLP-1, GIP	–	phase 1	undisclosed
Cpd86	Eli Lilly	GLP-1, GIP	–	preclinical	NA
ZP-DI-70	Zealand	GLP-1, GIP	–	preclinical	NA
Undisclosed	Takeda	GLP-1, GIP	–	undisclosed	NA
Undisclosed	MedImmune	GLP-1, GIP	–	undisclosed	NA
MAR423	Novo Nordisk / Marcadia	GLP-1, Gcg, GIP	acyl	preclinical	NA
Undisclosed	Sanofi	GLP-1, Gcg, GIP	–	undisclosed	NA
ZP-GG-23	Zealand	GLP-1, GLP-2	–	preclinical	NA
GUB09-123	Gubra	GLP-1, GLP-2	–	preclinical	NA

List of mixed agonists composed of proglucagon-derived peptides that are currently in preclinical development or in clinical evaluation. PSA, polysialic acid; PEG, polyethylene glycol.
[a]April 2016: decision not to advance into phase 3.
[b]April 2016: discontinued for undisclosed reasons.

engineered into the structure of the molecule with minimal change in chemical composition or biophysical properties, which may limit formation of anti-drug, neutralizing antibodies.

Three for All, All in One: Unimolecular Triagonists

GLP-1, GIP, and glucagon are endogenously hyper-secreted in response to bariatric surgery (Jiménez et al., 2013), suggesting that pharmacological simulation of this physiological response might lead to enhanced and safer restoration of normal body weight akin to a more physiological situation. Given the enhanced performance of the individual GcgR/GLP1R and GIPR/GLP-1R dual agonists in the treatment of obesity and T2D, as well as the structural similarity among the three peptides, we pursued the discovery of a unimolecular GcgR/GLP-1R/GIPR triple agonist. The structural optimization to achieve balanced, full-potency triple agonism within a single molecule of

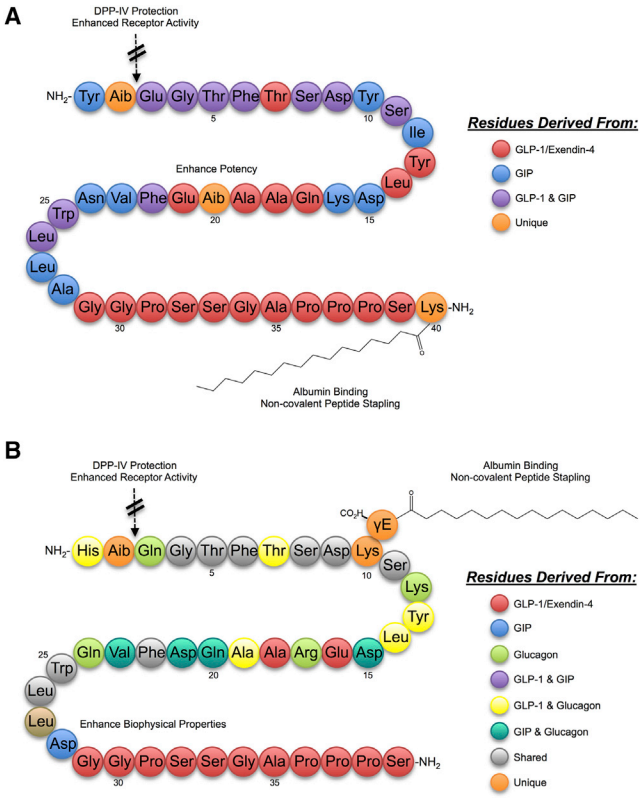

Figure 3. Structures of GIPR/GLP-1R Co-agonist and GcgR/GLP-1R/GIPR Triagonist

(A) Amino acid sequence of the acylated version of the GIPR/GLP-1R co-agonist (Finan et al., 2013). Residues derived from GLP-1 or exendin-4 are depicted in red, residues derived from GIP are depicted in blue, residues shared between GLP-1 and GIP are depicted in purple, and unique resides are depicted in orange. Aib, aminoisobutyric acid. A 16-carbon acyl chain (palmitoyl; 16:0) covalently attached through the side chain amine of Lys^{40} is represented with chemical structures.

(B) Amino acid sequence of the GcgR/GLP-1R/GIPR triagonist (Finan et al., 2015a). Residues derived from GLP-1 or exendin-4 are depicted in red, residues derived from GIP are depicted in blue, residues derived from glucagon are depicted in green, residues shared between GLP-1 and GIP are depicted in purple, residues shared between GLP-1 and glucagon are depicted in yellow, residues shared among all three native hormones are depicted in gray, and unique resides are depicted in orange. Aib, aminoisobutyric acid; γE, gamma glutamic acid. A 16-carbon acyl chain (palmitoyl; 16:0) covalently attached via a γ-carboxylate spacer to the side chain amine of Lys^{10} is represented with chemical structures.

composition similar to the native hormones was challenging, but eventually accomplished (Figure 3B) (Finan et al., 2015b). Only two positional modifications are not native to at least one of the native hormone sequences, which underscores the absence of any disruptive immunogenicity being observed in chronic study of these peptides. The two non-native changes impart enhanced pharmacokinetics by minimizing proteolytic digestion and extending plasma circulation time by promoting binding to plasma proteins. When administered to rodent models of obesity and diabetes, these triagonists more potently reversed the metabolic syndrome than any other reported agent. When specifically compared to the dual incretin co-agonists, these novel triple-acting peptides were superior in correcting the excess of adipose tissue mass, liver fat, food intake, and plasma cholesterol, while demonstrating increased energy expenditure, improved glucose tolerance, and protection from glucolipotoxic pancreatic islet destruction (Finan et al., 2015b). The relevant metabolic contribution of each of the three independent components within the triple agonist was demonstrated in genetic loss-of-function models for each of the three receptors (Finan et al., 2015b). Of particular significance, unlike the GIPR/GLP-1R dual agonist, the triagonist increased energy expenditure and endogenous circulating FGF21, which are contributed by the GcgR agonism component within the triagonist.

The successful in vivo validation of the GcgR/GLP-1R/GIPR triagonist confirms the previously unappreciated yet unique virtues of GcgR and GIPR agonism for the treatment and prevention of obesity and T2D (Scheen and Paquot, 2015). Triagonism offers a number of potential advantages that distinguishes it from either of the respective co-agonists. Of seminal importance is the prospect for greater weight loss in a broader population, with less risk of insufficient glucose control. Human T2D is far more heterogeneous than what is observed in rodent models. In patients with increased vulnerability to the diabetogenic potential of glucagon agonism, it is important to note that the addition of dual incretin action may prove to be far more protective of hyperglycemic risk than had been previously observed using only GLP-1 as a buffer. Although the preferred relative potency ratio in humans is currently unknown, the inclusion of dual incretin activity can allow for more aggressive constituent potency within the glucagon component to govern more weight loss. Of equal importance, most T2D patients would benefit from some degree of weight loss. The inherent glucagon action within the triagonist will drive energy expenditure and help burn calories to coincide with satiation effects delivered by dual incretin action, collectively providing greater potential for weight lowering.

Collectively, the recruitment of biochemical signaling through three receptors offers the potential to be less aggressive with any single component to achieve comparable metabolic results, while minimizing the risk for undesirable effects. However, all of these perceived virtues need to be documented in human clinical studies across broad populations. Nonetheless, it is clear that mono-agonism is relatively insufficient to correct the metabolic syndrome, in particular the excessive body weight found in the majority of patients. These mixed agonists provide reason for optimism. An additional corollary of this work is the established SAR within these engineered peptides. It provides the opportunity to precisely adjust the relative activities at each individual receptor to offer a portfolio of medicines with finely tuned mixed agonism. It is conceivable that a series of multi-agonists with a spectrum of pharmacological activities might yet emerge that offer differential health benefits suitable for use in different patients or within individual patients at different periods in disease regression to full health. In concert with emerging biomarkers, this might constitute more personalized metabolic medicine that could be achieved in a more precise and less empirical manner than the conventional approach in which therapy is currently delivered.

Expanding the Toolkit: Peptide-Based Steroid Delivery

The concept of poly-agonism is not limited to cellular receptors that biochemically signal by virtue of macromolecule binding. Quite separate from peptide- and protein-based metabolic

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medicines has been the broad-based pursuit of small molecules acting at nuclear hormone receptors for the treatment of multiple diseases. In fact, recent reports demonstrate that celastrol, a small-molecule tripterine with both antioxidant and transcriptional properties, can elicit comparable body weight lowering to the triagonist, albeit at much increased molar doses (Liu et al., 2015; Ma et al., 2015). Nuclear hormone-based medicines are some of the most important therapeutics ever discovered and include the likes of estrogen, testosterone, thyroid hormone, thiazolidinediones, and glucocorticoids. In addition to being highly effective, they unfortunately are in virtually all instances of narrow therapeutic index. This has promoted the search for selective agonists or agents with tissue-specific, biological properties, of which raloxifene, a selective estrogen receptor modulator (SERM), was precedent setting. To broaden the pharmacology that might lead to a more precise and personalized medicine, the possibility of integrating small- and large-molecule-based therapeutics was explored. Specifically, our hypothesis investigated whether potent steroid-based, nuclear hormone action could be selectively targeted to cells that express a unique extracellular peptide receptor to achieve tissue-selective pharmacology. Conceivably, this would maximize the metabolic benefits through concerted action while minimizing adverse effects by directing the steroid away from areas that do not express the peptide receptor. As proof of principle, the research began with the steroid hormone estrogen, which independently possesses potential for the treatment of metabolic diseases, but at the expense of gynecological and mitogenic risks.

Estrogen demonstrates leptin-like effects at the hypothalamus (Gao et al., 2007) and protects pancreatic islets from damage associated with metabolic disease (Tiano et al., 2011), thus coinciding with the pharmacology of GLP-1R agonists. To achieve synergistic metabolic effects among these two hormones at relevant cell populations, a set of chemically conjugated GLP-1 and estrogen receptor ligands was prepared (Finan et al., 2012). A chemically stable conjugate targets estrogen to cells via the GLP-1R, releasing it only in the intracellular compartment for subsequent interaction with its nuclear receptor. Treatment of diet-induced obese mice of both genders with this stable conjugate resulted in significantly enhanced body weight loss and improved glucose tolerance relative to GLP-1 alone. Notably, the enhanced body weight loss required a functional GLP-1R in the CNS. Importantly, these metabolic benefits occurred without any evidence of proliferation in reproductive tissues or promotion of cancer in tumor-bearing mice (Finan et al., 2012). In contrast, treatment with chemically labile conjugates where estrogen is untargeted and widely disseminated throughout systemic circulation did not provide any additional metabolic benefits relative to GLP-1 alone. Nonetheless the broad exposure elicited adverse effects consistent with systemic estrogenic activity, including uterine hypertrophy and accelerated growth in estrogen-sensitive MCF-7 xenograft tumors (Finan et al., 2012).

It remains to be determined to what extent any peptide co-agonist or peptide-steroid conjugate can cross the blood-brain barrier to reach deeper brain regions, or if the chemical modifications are influencing biodistribution to facilitate enhanced CNS localization. At the very least, hypothalamic areas not fully protected by the blood-brain barrier, such as the arcuate nucleus adjacent to the circumventricular organ and the median eminence, appear to be accessible and engaged by numerous protein-based hormones. Importantly, the enhanced metabolic benefits of stable GLP-1/estrogen conjugates were not simply a consequence of improved pharmacokinetics, as could be a consequence of conjugating a peptide with a more lipophilic steroid capable of binding albumin or other circulating chaperones. GLP-1-based conjugates with bile acids of a similar structure and lipophilic character as estrogen did not show an enhanced metabolic action profile. Consistent with this observation is the fact that the GLP-1/estrogen conjugates fractionally lost their weight-lowering efficacy in mice deficient for individual estrogen receptor subtypes and stimulate canonical estrogen receptor signaling in target tissues of wild-type mice (Finan et al., 2012).

This first proof-of-concept study illustrated that peptide-based delivery of a steroid hormone can enhance efficacy and safety. The conceptual approach is seemingly applicable to other macromolecules and conventional small-molecule combinations in other disease indications. Whether this method can be used to enhance the therapeutic potential of other nuclear hormones, such as those previously identified, remains a focus of ongoing research. This approach may transcend conventional small-molecule strategies, which have been burdened with molecular uncertainty to impart selective activity at specific nuclear receptor isoforms or convey tissue-divergent biology. Here, the challenge is identification of macromolecular ligands that contribute unique pharmacology and target a nuclear hormone to a tissue where it is capable of making an independent biological contribution. In the end, the fundamental objective of quality medicine is pharmacological performance, and the molecular nature of the drug is a secondary consideration. This opens a new avenue for conventional small-molecule medicinal chemistry, which historically has been restricted by the commercial demand for the enhanced convenience inherent to oral delivery.

Blockbuster, Conventional, or Precision Medicines?

The utility of these novel unimolecular combination therapeutics is currently being validated in clinical studies. These drug candidates, and the subsequent entities that they inspire, offer potential for an unparalleled medicinal impact on obesity and the associated co-morbidities that have reached epidemic prevalence. Specifically, the triagonist offers transformative potential due to its broad action profile and restorative efficacy built upon its molecular maturation integrated from two independent, beneficial co-agonists. Whether any of these drug candidates, and in particular the peptide-targeted nuclear hormones, are suited to broad patient populations or more select subgroups remains to be determined. While efficacy is of central importance, the question may be more a function of safety relative to the burden of disease. The most extreme forms of disease, where surgery is the only option, represent a different challenge than the more conventional T2D patient, where other medicinal options offer meaningful but variable performance. As a case in point, the GLP-1-conjugated estrogen impressively differentiated from therapy with either of the individual entities in rodents. Whether this can be replicated in human disease is a seminal uncertainty for all drug candidates,

but the unquantifiable risk that an oncogenic cell might be enhanced by double hormonal action of the honing peptide and the targeted nuclear hormone presents unique development challenges, and likely restricts initial considerations to limited patients. Importantly, the efficacy of various unimolecular combinations offers an alternative blueprint for the development of future precision medicines for the treatment of metabolic disease. With a similar advancement of biomarkers identifying distinct subpopulations of T2D and obesity, such as that of the GWAS-identified marker TCF7L2 (Chang et al., 2007; Grant et al., 2006), more individualized metabolic medicines can be envisioned. In summary, it appears not unlikely that there will be specific subpopulations of patients who would benefit more from one, rather than another, unimolecular co-agonist. Choice of the optimal therapeutic will depend on lead symptoms, complications, and comorbidities, among other factors. With an expanding portfolio of available compounds, it may, however, be the absence of reliable biomarkers identifying such subpopulations that represents a major obstacle on the way toward more personalized precision medicines for metabolic diseases.

Going forward, there are several reasons why this approach may not translate from relatively short-term pre-clinical rodent studies to chronic use in humans. While some of the obstacles are relatively straightforward to anticipate, the history of drug development is synonymous with failure for unexpected reasons. The macromolecular nature of these drug candidates is infrequently troubled by off-target toxicity resulting from poor drug disposition or unexpected metabolism that has plagued conventional small molecules. Nonetheless, large molecules present immunogenic risk, and these hybrid peptides possess non-native sequence and potential foreign structural epitopes that could prove problematic in certain patients, with the worst case scenario being the development of cross-reactive antibodies that neutralize endogenous hormones. More specific pharmacological concern pertains to GLP-1-based agonism, since each of these mixed agonisms possesses it to a certain degree. Cardiovascular effects such as elevated heart rate are well established and constitute one element to carefully monitor (Davies et al., 2011), as is concern of excess action at the exocrine pancreas (Butler et al., 2013) or the thyroid gland (Bjerre

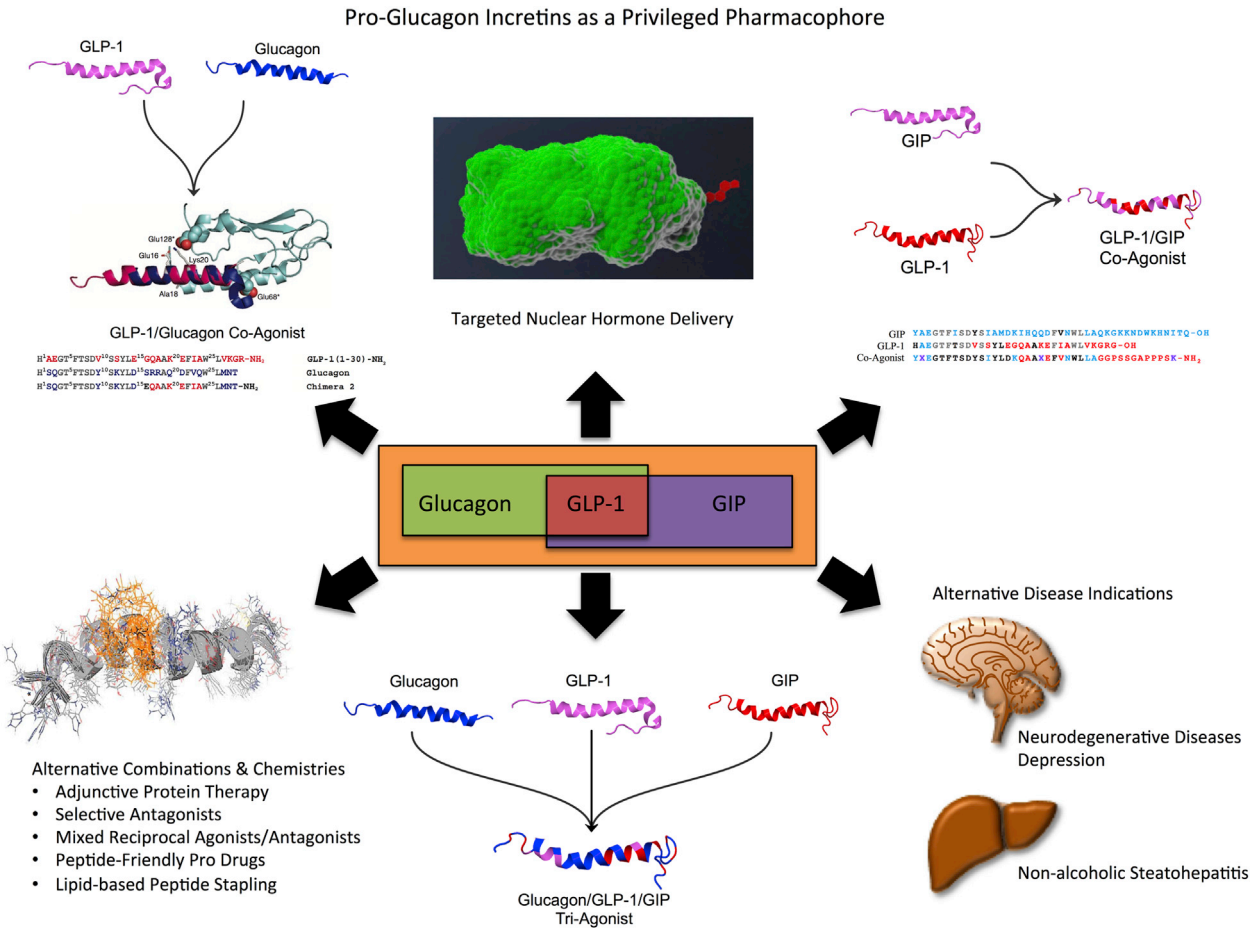

Figure 4. Glucagon Superfamily of Peptides as a Unique Pharmacophore
Different members of the glucagon superfamily of peptides have been incorporated into single molecules to generate novel medicines for the treatment of metabolic diseases, including sequence intermixed multi-agonists and peptide-nuclear hormone conjugates. Additional chemical engineering utilizing the glucagon superfamily of peptides has resulted in alternative combinations and chemistries that can be applied for academic and medicinal purposes. A unique feature of this family of peptides is that although structurally similar, many diverse actions are delivered by these exquisite hormones that could be harnessed for other disease indications such as neurodegenerative diseases, non-alcoholic steatohepatitis, and gastrointestinal disorders.

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Knudsen et al., 2010),which have been a controversial source of apprehension for chronic GLP-1R agonism that appears to have recently tempered. Whether the mixed agonists mitigate these risks or prove restricted to a point where efficacy is no greater than GLP-1 alone remains to be determined. Finally, while the referenced work has been integral in the repositioning of glucagon and GIP pharmacology, there is currently a deficiency of experience as to potential limitations in their chronic use. In this regard, it is best not to forget fenfluramine/phentermine (fen-phen), the combinatorial approach to the treatment of obesity that had initially delivered splendid results, but proved later to result in substantial heart valve damage (Connolly et al., 1997). Despite these perceived cautions, the glucagon superfamily of peptides is uniquely positioned to potentially serve as a privileged macromolecular pharmacophore, analogous to small molecules such as the serotonergic and adrenergic families, where multiple differentiated, valuable medicines have emerged. These endogenous glucagon-related peptides could yield an assembly of drugs armed with multiple different functionalities that could be applied in different disease indications, most notably neuronal protection (Duffy and Hölscher, 2013; Hansen et al., 2016; Ji et al., 2016) and gastrointestinal diseases (Schwartz et al., 2016) (Figure 4). The total count currently stands at three, with multiple registered medicines acting specifically at the glucagon, GLP-1, or GLP-2 receptors.

The future is now, and the prevalence of worldwide disease demands a diversity of approaches. If this were not demanding enough, recent epigenetic findings suggest that the current epidemic of metabolic disease may be programming enhanced consequences onto future generations. Time is of the essence, and interdisciplinary collaborations across academia, biotech, and the pharmaceutical industry are vital in recruiting creative alternatives in a setting where they can mature to enhance patient care. Independent of the immediate medicinal objective of these poly-agonists, these mixed agonists have contributed to the collective understanding of endocrine control of metabolism and have highlighted the danger in rendering pharmacological predictions based solely on individual genetically engineered mouse models. The iterative cycle of trial and error among rodent genetics, metabolic phenotyping, medicinal chemistry, and in vivo pharmacology is integrating ingredients needed to successfully discover therapeutics that can approach the efficacy currently obtained only through surgical procedures.

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